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CASE 4-31499A

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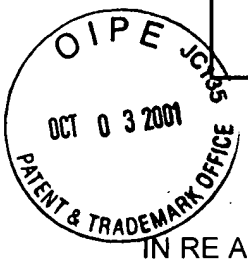

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF

BUEHLER ET AL.

#6

APPLICATION NO: 09/899,634

FILED: JULY 5, 2001

FOR: PCAR AND ITS USES

Assistant Commissioner for Patents
Washington, DC 20231

CLAIM OF PRIORITY UNDER 35 USC §119

Sir:

Applicants in the above-identified application hereby claim priority under the International Convention of Application No. 0016791.6, filed on July 7, 2000. This application is acknowledged in the Declaration of the instant case.

The certified copy of said application is submitted herewith.

Respectfully submitted,

Susan Hess

Susan Hess
Attorney for Applicants
Reg. No. 37,350

Novartis Corporation
Patent and Trademark Dept.
564 Morris Avenue
Summit, NJ 07901-1027
(908) 522-6938

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1. Your reference — **4-31499P1**

2. Patent application number *(The Patent Office will fill in this par* — **0016791.6**

10JUL00 E551381-1 000524
P01/7700 0.00-0016791.6

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

NOVARTIS AG
SCHWARZWALDALLEE 215
4058 BASEL
SWITZERLAND

Patent ADP number *(if you know it)* 7125487002

If the applicant is a corporate body, give the country/state of its incorporation — **SWITZERLAND**

4. Title of invention — **ORGANIC COMPOUNDS**

5. Name of your agent *(If you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

B.A. YORKE & CO.
CHARTERED PATENT AGENTS
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6. If you are declaring priority from one ore more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of filing (day/month/year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing (day/month/year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

Yes

a) any applicant named in part 3 is not an inventor, or

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Description **9**

Claim(s) **1**

Abstract

Drawing(s) **5(figures)** +5 SW

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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*

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Request for substantive examination *(Patents Form 10/77)*

Any other documents *(please specify)*

11. I/We request the grant of a patent on the basis of this application

Signature

B.A. Yorke & Co.

Date

07 July 2000

12. Name and daytime telephone number of person to contact in the United Kingdom

Mrs. E. Cheetham
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Organic Compounds

The invention provides improvements in the field of animal models for testing effects of genes introduced into animal cells or tissue by adenoviral gene transfer.

Adenoviruses infect cells using two cell surface receptors, the "Coxsackie B and adenovirus 2 and 5 receptor" (hereinafter referred to as CAR; Bergelson J.M., et al, Science 275, 1320-23, 1997) and the integrin receptors ($\alpha v\beta 3$ or $\alpha v\beta 5$; Wickham, T.J. et al, Cell 73, 309-19, 1993) the contents thereof being incorporated herein by reference. Adenoviral based vectors are widely used in gene therapy, as they represent one of the most efficient ways to deliver genes to target cells. They are of particular interest for in vivo gene therapy proof-of concept experiments in rodent models. However, rodent tissues are not well transducible with adenoviral vectors.

In its broad aspect the invention is concerned with genetic modification of target cells which are normally refractory to adenoviral transduction. More particularly the invention provides a plasmid construct that expresses a porcine adenovirus receptor (pCAR) and transgenic animals that show expression of pCAR.

Organ transplants of liver, kidney, lung and heart are now regularly performed as treatment for endstage organ disease. Despite the use of modern immunosuppressive drugs acute and chronic graft (tissue or organ) rejection still remain major factors in graft loss. There is, therefore, a continued need for means to inhibit acute and chronic graft rejection and increase graft acceptance, e.g. through induction of peripheral tolerance without causing serious toxic side effects typically associated with conventional immunosuppressant therapy. When considering cell transplantation, e.g. bone marrow derived cells, islet cells, neuronal cells etc. one is faced with similar problems of rejection. Making organs or cells less immunogenic through genetic modification is seen as an alternative or add on to conventional immunosuppression.

Rodent animal models are of crucial importance for testing the immunomodulatory effects of new gene products. However in the case of using adenovirus as gene delivery vehicle rodent models have so far proven to be of limited value, as many rodent organs or cell types are refractory to adenoviral transduction. This may be due to the fact that either the adenoviral

receptor CAR is not expressed or only weakly expressed on the cell surface of the cells of interest.

Accordingly, the invention provides a plasmid or vector construct that comprises a DNA molecule which expresses porcine CAR (hereinafter referred to as pCAR) or a biologically active fragment or derivative thereof, for example a C-terminally truncated porcine CAR (hereinafter referred to as ΔpCAR), that retains full functionality as adenoviral receptor.

pCAR comprises an intracellular domain, a transmembrane domain and a an extracellular domain that binds to the adenoviral fibre proteins, i.e. a total sequence of 365 amino-acids. It will be understood that any nucleic acid sequence encoding a porcine CAR homologue is a candidate for utilization in the present invention. For example, it may include a pCAR sequence with a modified, mutated or truncated region thereof, that retains the activity of mediating adenoviral transduction. It will be further understood by the skilled person that any nucleic acid sequence which encodes a biologically active form of pCAR, including but not limited to a genomic or cDNA sequence or functionally equivalent variant or mutant thereof or a fragment thereof which encodes a biologically active protein fragment or derivative which mediates adenoviral transduction, may be utilized in the present invention. For example, ΔpCAR may comprise the leader sequence of 19 amino-acids, the extracellular domain of 216 amino-acids, the transmembrane domain of 24 amino-acids and a truncated cytoplasmic domain, e.g. limited to 3 amino-acids. Two potential sites for N-glycosylation are located at Asn 106 and Asn 201. Amino-acids present in the sequence which are not essential to the activity may be changed by mutation, e.g. amino-acid 258 may be changed from Val to Ile; amino-acid 262 may be changed from His to Arg.

Preferred nucleic acid sequence for use in the invention is e.g. as disclosed in Figure 2 from nucleotide 3229 to nucleotide 4014. The corresponding amino acid sequence encoded by such DNA sequence is indicated in Figure 1.

Any known expression vector or plasmid that is capable of expression upon transfection of a specified eukaryotic target cell may be utilized to pratice the invention. "Plasmid" and "vector" can be used interchangeably in the present specification as the plasmid is the most commonly used form of vector. An expression vector is a vector capable of directing the expression of genes to which they are operatively linked. An operable linkage as used herein

refers to the position, orientation and linkage between a structural gene and expression control element(s) such that the structural gene can be expressed in any host cell. The term "expression control element" includes promoters, enhancers, ribosome binding sites etc. Any eukaryotic promoter and/or enhancer sequences available to the skilled person which are known to control expression of the nucleic acid of interest may be used in plasmid vector constructs, including but not limited to a cytomegalovirus (CMV) promoter, a Rous Sarcoma (RVS) promoter, a Murine Leukemia (MLV) promoter, a herpes simplex virus (HVS) promoter, such as HSV-tk, a β-actin promoter, e.g. chicken β-actin, as well as any additional tissue specific or signal specific regulatory sequence that induces expression in the target cell or tissue of interest. A preferred expression vector or plasmid according to the invention is e.g. an eukaryotic expression vectors, e.g. a pβ-actin-p16PL vector such as p(chicken)β-actin-p16PL.

In one such embodiment, a DNA sequence encoding pCAR is subcloned into the DNA plasmid expression vector, e.g. pβ-actin-p16PL, resulting in pβ-actin-pCAR-p16PL. p16PL is a standard mammalian expression vector, containing a gene that encodes a selectable marker, e.g. an antibiotic resistance gene, and a β-actin promoter active in mammalian cells (K. M. Marsden et al, J. Neurosc., May 15, 1996, 16(10): 3265-3273). Such a construct, which may be constructed by one of ordinary skill with components available from numerous sources, will drive expression of a pCAR DNA fragment ligated downstream of the β-actin promoter subsequent to transfection of the target cell. More specifically, pCAR is cloned from pig liver RNA using a PCR based approach. The PCR fragment is inserted into the expression vector pSport (Life Technologies). This plasmid serves as template to create the truncated version of ΔpCAR. Preferably pβ–actin is pβ-(chicken) actin.

The invention further provides host cells into which a recombinant expression vector of the invention has been introduced. A host cell can be any prokaryotic or eukaryotic cell, e.g. bacterial such as E. Coli, yeast or mammalian cells, e.g. CHO or COS cells.

The host cells of the invention may preferably be used to produce nonhuman transgenic animals, preferably a mammal, more preferably a rodent such as a rat or mouse, or a pig. For example, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which a pCAR-coding sequence has been introduced. A transgenic animal of the invention, e.g. a rodent or a pig, may be created by introducing a pCAR expression construct into the

male pronuclei of a fertilized oocyte, e.g. by microinjection, or into embryonic stem cells, e.g. by electroporation. Methods for generating transgenic rodents have become conventional in the art and are described e.g. in USP 4,736,866, 4,870,009, 4,873,191, or in Manipulating the Mouse Embryo, B. Hogan, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). For example the expression construct may be introduced into an embryonic stem cell line and cells in which the introduced pCAR gene has integrated are selected. The selected cells are then used to produce chimaeras with known standard procedures. A chimaeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. The pCAR expression plasmid may also be inserted into somatic/body cells of the donor animal to provide a somatic recombinant animal, from whom the DNA construct is not capable of being passed on to offspring. For example, a somatic cell from the transgenic animal can be isolated and induced to exit the growth cycle and enter G_o phase. The quiescent cell can then be fused, e.g. through the use of electrical pulses, to an enucluated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyst and then transferred to pseudopregnant female foster animal. The offspring of this female foster animal will be a clone of the animal from which the somatic cell is isolated.

The present invention also provides a method for improving adenoviral gene transfer in a rodent using a transgenic rodent which expresses or overexpresses pCAR. Such rodents may be used as models in gene therapy to test adenoviral transduction, e.g. prevention or treatment of acute or chronic graft rejection, autoimmune disorders, e.g rheumatoid arthritis, cardiovascular disorders, e.g. restenosis, nervous sytem disorders, e.g. parkinson disease, etc.. A preferred embodiment of the invention is the use of such rodents expressing or overexpressing pCAR in transplantation experiments, for example, of organs, tissues or cells, e.g. lung, heart, kidney, liver, pancreas, small bowel, spleen, pancreatic islets, neuronal or stem cells, etc. For example, organs, tissues or cells of such transgenic rodents, e.g. mice, are removed, in vitro transduced with the adenoviral gene delivery vector to be tested and then transplanted into rodents, e.g. mice, e.g. such animals which do not express pCAR.

The functional expression of pCAR, e.g. ΔpCAR may also be used to generate transgenic pigs that overexpress this adenoviral receptor. Porcine organs, tissues or cells transgenically

modified to express high levels of pCAR may be used as recipients for adenoviral gene therapy vectors. Such transgenic modified organs, tissues or cells can be transfected with adenoviral gene therapy vectors carrying thrapeutically beneficial genes either ex vivo or in vivo and can be subsequently transplanted in a recipient. Beneficial genes are those that are expected to confer graft protection following transplantation of these gene delivered organs in xenotransplantation therapy. The present invention comprises a method to generate such transgenic pigs expressing high levels of pCAR or a functionally equivalent variant or mutant thereof or a fragment thereof, e.g. as disclosed above, and gene therapy methods for preventing or inhibiting graft rejection in a recipient using organs, tissues or cells of such transgenic pigs.

The following Exampes are illustrative only and not limiting of the invention. The β-actin promotor used in the Examples is the β-(chicken)actin promotor.

Example 1: Construction of the expression vector (Figure 2)

The full length cDNA for porcine CAR is cloned from pig liver using degenerated primers (forward: 5'-accatggcgckcctrctgt-3' and reverse: 5'-catatggaggctytatacya-3' in which k=g or t; r=a or g and y=c or t). The PCR fragment is bluntend inserted into the vector pSport (Life Technologies). Porcine CAR (Figure 3) has an overall aminoacid homology of 91% to human as well as mouse CAR. This clone is used as template to generate the ΔpCAR gene as disclosed in Figure 2 from nucleotide 3229 to nucleotide 4014, using PCR. The primers used to generate this construct contain two amino acid changes at the C-terminal end of the construct. The sense primer SpeI-CAR (5'–ggactagtgccaccatggcgctcctgctgtgcttc–3') is located at position 1-21 of pCAR and contains a SpeI site, a Kozak sequence and the start codon. The antisense primer CAR-XbaI (5'–gctctagattaacgacagcaaaagatgataagacc–3') is located at position 760-786 of porcine CAR containing a stop codon and a XbaI site. The PCR amplification used the following conditions: 1x native *Pfu* buffer, 2.5mM $MgCl_2$, 0.2mM dNTPs, 2.5 U native *Pfu* polymerase (Stratagene) and 20pmol SpeI-CAR and CAR-XbaI (each). Porcine CAR cDNA (5ng) is used as template and hot start PCR is performed using the following profile: 1x (5min 95°C) 20x (30sec 95°C, 1min 55°C, 1min 30sec 72°C) 1x (3min 72°C). A PCR product of a predicted size of 788bps is obtained and separated on a 1% low melting agarose gel (SeaPlaque GTG; FMC). The band is excised and the PCR product isolated from the gel piece using the QIAquick gel extraction kit from Qiagen

according to the manufacturers protocol. The isolated PCR product is then digest with *XbaI* (LifeTechnologies) and repurified as described above. The digested purified PCR product is ligated into *MscI-XbaI* digested pβactin-16PL vector.

INVaF'chemically ultracompetent bacteria from Invitrogen are transformed and 48 colonies picked, rescreened by PCR using SpeI-CAR and CAR-XbaI as primers. From 48 colonies analyzed 20 contain the insert – 12 are selected for DNA sequencing. The sequencing primer actinsense (5'-accggcggggtttatatcttc–3') is the 5'-primer located just upstream of the MCS of the pβactin-16PL vector. Actinanti (5'-cctctacagatgtgatatggc–3') is the 3'-primer located just downstream of the MCS of pβactin-16PL vector. The nucleotide sequence of the β-actin promotor, the ΔpCAR gene and the SV40 polyadenylation signal is shown in Figure 2.

Example 2: In vitro expression of ΔpCAR in mammalian cells (Western blot)

A human lung carcinoma cell (A30), rat embryonic fibroblasts (Rat2, ATCC:CRL-1764) and chinese hamster ovary cells (CHO) are used for transient transfections. Culture conditions are as follows:

Cell Line	Medium	Serum	Supplement	Antibiotics
A30	RPMI	10%FBS	1%NEAA	1%PS
Rat2	DMEM	10%FBS		1%PS
CHO	αMEM	10%FBS		1%PS

In addition, all media contain 2mM Glutamax II. Cultures are maintained at 37°C in a water saturated air atmosphere containing 5%CO_2.
Cells are transfected with either the control plasmid (pβactin-16PL vector) or pβactin –ΔpCAR–16PL. In brief, an 80% confluent (approx. $1x10^8$ cells) 15cm dish is transfected with 15μg plasmid DNA using SuperFect from Qiagen according to the manufacturer's protocol. After 24h, cells are harvested, washed and cell pellet resuspended in 0.5ml Lämmli's buffer. Western blotting supplies are obtained from BioRad unless otherwise stated. Samples are sonicated for 10sec, heat-denatured for 5min at 95°C and cellular debris removed by centrifugation (10min 13krpm Eppendorf). Samples are stored at –20°C until further use. A quantity of 30μl/lane is loaded on to a 12% denaturing polyacrylamide gel (SDS-PAGE) and run at 100V for 90min in 1xTris/Glycine/SDS buffer.

Gel is then electrotransfered onto a 0.45µm Protan BA85 (Schleicher&Schuell) nitrocellulose membrane in 1xTris/Glycine buffer (Novex) containing 20% methanol. The membrane is blocked for 1h in phosphate-buffered saline (PBS) containing 5% non-fat dry milk and 1%Tween 20 (Sigma), followed by 1h incubation with an affinity-purified polyclonal chicken-anti human CAR antibody at 1:500 in blocking solution. In between antibody incubation steps the membrane is washed by two short rinses in PBS/1%Tween 20 followed by 2x15min in the same washing buffer. The membrane is incubated for 1h with a biotinylated rabbit-anti chicken IgY (Vector Laboratories) diluted at 1:1000 in blocking solution, followed by 30min incubation with streptavidin-horseradish peroxidase (Vector Laboratories) at 1:1000 in blocking solution. Membrane is incubated for 5min in enhanced chemiluminescence (ECL) substrate (Amersham), solution is carefully drained and membrane put in a Photogene Development folder (Life Technologies). ECL signals are detected by exposing Hyperfilm ECL (Amersham) to the membrane and films are developed on a X-Ray film developer (Agfa).

All 3 different cell lines which are transfected with ΔpCAR–16PL show an additional strong protein band which has the predicted molecular size. As a positive control 100 ng of recombinant human soluble CAR (hCAR) purified from E.coli source is used.

The polyclonal chicken-anti human CAR antibody used above are prepared as follows:
A soluble version of human CAR is generated by PCR using the CAR1 (5'-accggccatggcatatggatttcgccagaa-3' and the CAR2 (5'-accggctcgagagctttatttgaaggagggac-3') primers. As template full length human CAR cloned from HeLa cells is used. The soluble human CAR PCR fragment is digested with Nde1 and Xho1 and inserted into the prokaryotic expression vector pET-17H, which contains a C-terminal histidine tag. The construct is transformed into bacteria and cells are induced to produce the soluble human CAR protein. The protein is purified by commonly used methods and is injected into an adult female chick. The eggs of the hen are collected and antibodies isolated from the egg yolk.

Example 3: Functionality of ΔpCAR in mammalian cells (adenoviral gene transfer)

The functionality is tested by transient transfection of CHO cells with the construct to be tested or the control plasmid, followed by transduction with an adenovirus which contains a reporter gene.
CHO cells are seeded into 24 well plate at a density of 12'000 cells/well. Cells are transiently

transfected with 0.5μg plasmid DNA of either pβactin-16PL or pβactin-ΔpCAR-16PL and incubated for 24h. Cells are then transduced with an adenoviral vector carrying β-galactosidase as a reporter gene (moi 0-100) for 2h. Virus solution is removed and cells incubated for an additional 4 days. Reporter gene expression is monitored using staining for nuclear β-galactosidase. Only ΔpCAR transfected cells are transduced with the reporter gene.

Example 4: Generation of transgenic mice

(a) Generation of ΔpCAR BALB/c ES cell lines

$5x10^6$ BALB/c ES cells ("Efficient targeting of the IL-4 gene in a BALB/c embryonic stem cell line", Noben-Trauth *et al.*, Transgenic-Res. 1996 Nov; 5(6): 487-91) are electroporated with 30 μg of the linearized construct. Transfected cells are selected with G418 (200 μg/ml). G418-resistant clones are screened for integration events by PCR. The ES cells are lysed 1h/37°C with 20 μl Lysis buffer (PCR buffer 1X; SDS 1.7 μM; Proteinase K 50 μg/ml) heat inactivated 85°C/15 Min. and cleared by centrifugation. 1,3 μl lysed solution is used in for a 50 μl PCR. Positive clones are further verified by Southern analysis.

(b) Generation of ΔpCAR transgenic mice

BALB/c-ES cell clones carrying one ΔpCAR allele are injected into C57BL/6 host blastocysts and transferred into pseudopregnant foster mothers according to standard protocols. Chimaeras are mated with BALB/c females and albino offspring (indicative for germ line transmission) are analyzed by PCR for target integration and Southern analysis. Heterozygous animals are generated by back-crossing of F1 animals to Balb/c wild type animals and Southern analysis of the F2 animals. The homozygous lines are established by mating heterozygous F1 animals.

Example 5: Transplantation

Hearts of transgenic mice obtained according to Example 4 are removed, in vitro transduced by infusion with an adenovirus carrying β-galactosidase and then heterotopically transplanted into female mice (which do not express pCAR). Age matched Balb/c male mice are used as controls. 4 days after transplantation hearts are removed, perfusion stained for nuclear β-galactosidase, paraffin embedded and sectioned. Sectiones are counterstained

with hematoxylin and evaluated by light microscopy. Positive expression for β-galactosidase is seen in the transgenic mice compared to the control animals.

CLAIMS

1. C-terminally truncated porcine CAR or a fragment or derivative thereof which mediates adenoviral transduction.

2. C-terminally truncated porcine CAR as disclosed in Figure 1 or a fragment or derivative thereof which mediates adenoviral transduction.

3. A DNA sequence which encodes a C-terminally truncated porcine CAR according to claim 1 or 2.

4. A plasmid or vector construct that comprises a DNA molecule which expresses a porcine CAR or a fragment or derivative thereof which mediates adenoviral transduction.

5. A plasmid or vector construct that comprises a DNA which expresses a C-terminally truncated porcine CAR according to claim 1 or 2.

6. A plasmid or vector, substantially as hereinbefore defined or described, a process for its preparation and its uses, substantially as hereinbefore defined or described.

7. Host cells into which a vector according to any one of claims 4 to 6 has been introduced.

8. A method for generating non human transgenic animals expressing or overexpressing a porcine CAR, substantially as hereinbefore defined or described.

9. A method for improving adenoviral gene transfer in a non human animal, substantially as hereinbefore defined or described.

10. A method for improving gene therapy, substantially as hereinbefore defined or described.

11. C-terminally truncated porcine CAR according to claim 1, a DNA sequence according to claim 2, a plasmid or vector according to any one of claims 4 to 6, host cells according to claim 7, for use to improve adenoviral gene transfer.

Figure 1

```
  1 MALLLCFVLLCGVADLTRSLSITTPEQMIEKAKGETAYLPCRFTLGPEDQ  50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 MALLLCFVLLCGVADLTRSLSITTPEQMIEKAKGETAYLPCRFTLGPEDQ  50

 51 GPLDIEWLLSPADNQKVDQVIILYSGDKIYDDYYQDLKGRVHFTSNDLKS 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 GPLDIEWLLSPADNQKVDQVIILYSGDKIYDDYYQDLKGRVHFTSNDLKS 100

101 GDASINVTNLQLSDIGTYQCKVKKAPGVGNKKIQLTVLLKPSGTRCYVDG 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 GDASINVTNLQLSDIGTYQCKVKKAPGVGNKKIQLTVLLKPSGTRCYVDG 150

151 SEEIGNDFKLKCEPKEGSLPLLYEWQKLSNSQKLPTLWLAEMTSPVISVK 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 SEEIGNDFKLKCEPKEGSLPLLYEWQKLSNSQKLPTLWLAEMTSPVISVK 200

201 NASTEYSGTYSCTVKNRVGSDQCLLRLDVVPPSNRAGTIAGAVIGVLLAL 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 NASTEYSGTYSCTVKNRVGSDQCLLRLDVVPPSNRAGTIAGAVIGVLLAL 250

251 VLIGLIIFCCR*..................................... 262
    |||||||:|||
251 VLIGLIVFCCHKKRREEKYEKEVHHDIREDVPPPKSRTSTARSYLGSNHS 300
```

Figure 2

DNA sequence of ΔpCAR Fragment:

Total length:	4286 bps
1-3186	chicken β-actin promotor
3229-4014	ΔpCAR gene
4020-4260	SV40 polyadenylation signal

```
        10         20         30         40         50         60         70
CGGTGCGGGC CTCTTCGCTA TTACGCCAGC TGGCGAAAGG GGGATGTGCT GCAAGGCGAT TAAGTTGGGT

        80         90        100        110        120        130        140
AACGCCAGGG TTTTCCCAGT CACGACGTTG TAAAACGACG GCCAGTGCCA AGTTGGGATC TTTGCATTGG

       150        160        170        180        190        200        210
CCCACGGCTC TCAGGATGGG GATGCTCCCC TTCAGCACCC GGTTCCCCTT GGAAACTGAT GGTCCTGGCT

       220        230        240        250        260        270        280
CTGTGGCATG GCAGTGGCAC TGTGAGGAGC CCCTACCAGC AGCACACAGT GGGTTTGGCA CTGCCACGCT

       290        300        310        320        330        340        350
CCGGATGCCG CGCTCTGATC CAACCCCATA ATCAAGGGAA CCCGAATTGC CCCATCATTG CCCCCACCAC

       360        370        380        390        400        410        420
CCCCATCCTG CCGGGCCCTC ACACCCCACG CTGCCTTGTG GTGACATTCC CCAGCCCAAA CCCACGGCTT

       430        440        450        460        470        480        490
CATGGCTACC GCGGGGCATT TCCCATTGCC GCCCCATTAT CAGCTCTGCA CACCTCCCGC TGTACCCATG

       500        510        520        530        540        550        560
CCTCGTGGCT GCCCTTCTTT GACGTATAAT CTTCTAATTA ATACCCGGCC TTGTCAAAGT GGAGCACAAA

       570        580        590        600        610        620        630
CGTTAATTAA TTCCCCAGCA GGCAGGTAAT TAACAGTGTG ACTCCCTTTT TGCTGCGAGT GGGGCTGATA

       640        650        660        670        680        690        700
CAGAGAGATG TGGCACTATG GAGCCCACGG GGTCCTGGCA CTGGGTGCCC ACGGAGGTCC CCATGTGCTG

       710        720        730        740        750        760        770
CAGTGTCACC GCCTCCGAGG TGACAGTATT GTCCCTGCGG TGTCCCTGCA GCTCAGCTCT GTCCACAGGG

       780        790        800        810        820        830        840
CCACCTCCAG TTTGGAGGGG ACACAATGCA GCCCCGATGC AACCCATCCT CGCAGCATCC CAGGGACAAA

       850        860        870        880        890        900        910
GACCCCACTG CAAGACCGCA CACAGGGCTG GGTCCCGCTC CCCTAATATC TACAGTGCTT TTGCATGGCC

       920        930        940        950        960        970        980
CCTTAATCAA TGCAGTTAAT CAGCATGCGC TCATGCACCG CTCTGGAGCT GCAAAGCCCC TCGCAGCGCT

       990       1000       1010       1020       1030       1040       1050
GCTCACCAAC ACCGCGCACC GCCCCGGCCC AGCCTGCAGC ACGCGCTGCA AACAGGAAAG AAACAAAATA
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      1060       1070       1080       1090       1100       1110       1120
TTGCCCAAAT GTAGGCAAAG GCATTCGGCT GCCTTGACCT CCGCCGGGCC GGGCCCTGCC TGACTCAGCT

      1130       1140       1150       1160       1170       1180       1190
CCTTACTCAG CGCTCGCTTC CTCCCTCCGG CTGCCACCGC CGCAGCGCAC ACCCTGACAA AGAGTGGCCC

      1200       1210       1220       1230       1240       1250       1260
TTAACGGGCT CTGAGGTGCA CCCAGCAGTG CACTCAGCAG TCCAAGGGCC GGCCTGGAGG TTTGCACCGC

      1270       1280       1290       1300       1310       1320       1330
TACGTGCTGA CATTAGCATT GAACTTGGCC CTGGGTAGTG CTGCAGGCCG GGCGGGGTGG GTGTAGAGAG

      1340       1350       1360       1370       1380       1390       1400
TGCAGCGCGC GTTGCACCCG GTGCCCCTTC CCCTCCCTTG CATCCCAGCA GGCTGCACCC CAGCACCAGG

      1410       1420       1430       1440       1450       1460       1470
CCCGTGCATG CATGCTCCTG GTGTTATTGC AGCCTGGTGC ATGCATGCGT CTTAGTGGTG CAGCGCTGTG

      1480       1490       1500       1510       1520       1530       1540
CATGCATCCT CCTTGGTGTG TAGCAGCTTA GTGCATGCAT ACCCCTCGGT GTTATTGCTG CTCTGTGCAC

      1550       1560       1570       1580       1590       1600       1610
GCACGCTCAT TGTATCACTT CATCCCAGTG CATGCACTCA CACTGGAGCG ATTGCTGCTC GGTGCACGCA

      1620       1630       1640       1650       1660       1670       1680
CACTCATTGT ATCACGTCAG CTCAGTGGCT GCACGCACAC CGGTGTTATT GCTGCTCGGT GCGTGCATGC

      1690       1700       1710       1720       1730       1740       1750
ACATCAGTGT CGCTGCAGCT CAGTGCATGC ACGCTCATTG CCCATCGCTA TCCCTGCCTC TCCTGCTGGC

      1760       1770       1780       1790       1800       1810       1820
GCTCCCCGGG AGGTGACTTC AAGGGGACCG CAGGACCACC TCGGGGGTGG GGGGAGGGCT GCACACGCGG

      1830       1840       1850       1860       1870       1880       1890
ACCCCGCTCC CCCTCCCCAA CAAAGCACTG TGGAATCAAA AAGGGGGGAG GGGGGATGGA GCGGCCCGTC

      1900       1910       1920       1930       1940       1950       1960
ACACCCCCGC CCCACACCCT CACCTCGAGG TGAGCCCCAC GTTCTGCTTC ACTCTCCCCA TCTCCCCCCC

      1970       1980       1990       2000       2010       2020       2030
CTCCCCACCC CCAATTTTGT ATTTATTTAT TTTTTAATTA TTTTGTGCAG CGATGGGGGC GGGGGGGGGG

      2040       2050       2060       2070       2080       2090       2100
GGGGCGCGCG CCAGGCGGGG CGGGGCGGGG CGAGGGGCGG GGCGGGGCGA GGCGGAGAGG TGCGGCGGCA

      2110       2120       2130       2140       2150       2160       2170
GCCAATCAGA GCGGCGCGCT CCGAAAGTTT CCTTTTATGG CGAGGCGGCG GCGGCGGCGG CCCTATAAAA

      2180       2190       2200       2210       2220       2230       2240
AGCGAAGCGC GCGGCGGGCG GGAGTCGCTG CGTTGCCTTC GCCCCGTGCC CCGCTCCGCG CCGCCTCGCG

      2250       2260       2270       2280       2290       2300       2310
CCGCCCGCCC CGGCTCTGAC TGACCGCGTT ACTCCCACAG GTGAGCGGGC GGGACGGCCC TTCTCCTCCG

      2320       2330       2340       2350       2360       2370       2380
GGCTGTAATT AGCGCTTGGT TTAATGACGG CTCGTTTCTT TTCTGTGGCT GCGTGAAAGC CTTAAAGGGC

      2390       2400       2410       2420       2430       2440       2450
TCCGGGAGGG CCCTTTGTGC GGGGGGGAGC GGCTCGGGGG GTGCGTGCGT GTGTGTGTGC GTGGGGAGCG

      2460       2470       2480       2490       2500       2510       2520
CCGCGTGCGG CCCGCGCTGC CCGGCGGCTG TGAGCGCTGC GGGCGCGGCG CGGGGCTTTG TGCGCTCCGC

      2530       2540       2550       2560       2570       2580       2590
GTGTGCGCGA GGGGAGCGCG GCCGGGGGCG GTGCCCCGCG GTGCGGGGGG GCTGCGAGGG GAACAAAGGC

      2600       2610       2620       2630       2640       2650       2660
TGCGTGCGGG GTGTGTGCGT GGGGGGGTGA GCAGGGGGTG TGGGCGCGGC GGTCGGGCTG TAACCCCCCC

      2670       2680       2690       2700       2710       2720       2730
CTGCACCCCC CTCCCCGAGT TGCTGAGCAC GGCCCGGCTT CGGGTGCGGG GCTCCGTGCG GGGCGTGGCG
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      2740        2750        2760        2770        2780        2790        2800
CGGGGCTCGC CGTGCCGGGC GGGGGGTGGC GGCAGGTGGG GGTGCCGGGC GGGGCGGGGC CGCCTCGGGC

      2810        2820        2830        2840        2850        2860        2870
CGGGGAGGGC TCGGGCGAGG GGCGCGGCGG CCCCGGAGCG CCGGCGGCTG TCGAGGCGCG GCGAGCCGCA

      2880        2890        2900        2910        2920        2930        2940
GCCATTGCCT TTTATGGTAA TCGTGCGAGA GGGCGCAGGG ACTTCCTTTG TCCCAAATCT GGCGGAGCCG

      2950        2960        2970        2980        2990        3000        3010
AAATCTGGGA GGCGCCGCCG CACCCCCTCT AGCGGGCGCG GGCGAAGCGG TGCGGCGCCG GCAGGAAGGA

      3020        3030        3040        3050        3060        3070        3080
AATGGGCGGG GAGGGCCTTC GTGCGTCGCC GCGCCGCCGT CCCCTTCTCC ATCTCCAGCC TCGGGGCTGC

      3090        3100        3110        3120        3130        3140        3150
CGCAGGGGGA CGGCTGCCTT CGGGGGGGAC GGGGCAGGGC GGGGTTCGGC TTCTGGCGTG TGACCGGCGG

      3160        3170        3180        3190        3200        3210        3220
GGTTTATATC TTCCCTTCTC TGTTCCTCCG CAGCCCCCAA GCTTAAGGTG CACGGCCCAC CTGGGGACTA

      3230        3240        3250        3260        3270        3280        3290
GTGCCACCAT GGCGCTCCTG CTGTGCTTCG TGCTCCTGTG CGGAGTCGCG GATCTCACCA GAAGTTTGAG

      3300        3310        3320        3330        3340        3350        3360
TATCACTACT CCTGAACAGA TGATTGAAAA GGCCAAGGGG GAAACTGCCT ATTTGCCATG CAGATTTACC

      3370        3380        3390        3400        3410        3420        3430
CTGGGTCCAG AAGACCAGGG GCCGCTGGAC ATCGAGTGGC TGCTGTCACC AGCTGATAAT CAGAAGGTGG

      3440        3450        3460        3470        3480        3490        3500
ATCAAGTGAT TATTTTATAT TCTGGAGACA AAATTTATGA CGACTACTAC CAAGATCTGA AAGGACGAGT

      3510        3520        3530        3540        3550        3560        3570
ACATTTTACA AGTAATGATC TCAAATCTGG TGATGCATCA ATAAATGTAA CAAATCTACA GTTGTCAGAT

      3580        3590        3600        3610        3620        3630        3640
ATTGGCACAT ATCAGTGCAA AGTGAAAAAG GCTCCTGGTG.TTGGAAATAA GAAGATTCAG CTGACAGTTC

      3650        3660        3670        3680        3690        3700        3710
TTCTTAAGCC TTCAGGTACA AGATGTTATG TTGATGGATC AGAAGAAATT GGAAATGACT TTAAACTAAA

      3720        3730        3740        3750        3760        3770        3780
ATGTGAACCA AAAGAAGGTT CACTCCCATT ACTATATGAA TGGCAGAAAT TGTCCAATTC ACAGAAGCTG

      3790        3800        3810        3820        3830        3840        3850
CCCACCTTGT GGTTAGCAGA AATGACTTCA CCTGTTATAT CTGTAAAAAA TGCCTCTACT GAATACTCTG

      3860        3870        3880        3890        3900        3910        3920
GGACATACAG CTGTACCGTG AAAAACAGAG TGGGCTCTGA TCAATGCCTG CTTCGCCTGG ATGTGGTTCC

      3930        3940        3950        3960        3970        3980        3990
TCCTTCAAAT AGAGCTGGAA CAATTGCAGG AGCTGTTATA GGAGTTTTGC TTGCTCTAGT GCTCATTGGT

      4000        4010        4020        4030        4040        4050        4060
CTTATCATCT TTTGCTGTCG TTAATCTAGA TAAGTAATGA TCATAATCAG CCATATCACA TCTGTAGAGG

      4070        4080        4090        4100        4110        4120        4130
TTTTACTTGC TTTAAAAAAC CTCCCACACC TCCCCCTGAA CCTGAAACAT AAAATGAATG CAATTGTTGT

      4140        4150        4160        4170        4180        4190        4200
TGTTAACTTG TTTATTGCAG CTTATAATGG TTACAAATAA AGCAATAGCA TCACAAATTT CACAAATAAA

      4210        4220        4230        4240        4250        4260        4270
GCATTTTTTT CACTGCATTC TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGGATCC

      4280
CCGGGTACCG AGCTCG
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Figure 3

DNA Sequence of full length porcine CAR: Total length:1098bp

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   1  ATGGCGCTCC TGCTGTGCTT CGTGCTCCTG TGCGGAGTCG CGGATCTCAC
  51  CAGAAGTTTG AGTATCACTA CTCCTGAACA GATGATTGAA AAGGCCAAAG
 101  GGGAAACTGC CTATTTGCCA TGCAGATTTA CCCTGGGTCC AGAAGACCAG
 151  GGGCCGCTGG ACATCGAGTG GCTGCTGTCA CCAGCTGATA ATCAGAAGGT
 201  GGATCAAGTG ATTATTTTAT ATTCTGGAGA CAAAATTTAT GACGACTACT
 251  ACCAAGATCT GAAAGGACGA GTACATTTTA CAAGTAATGA TCTCAAATCT
 301  GGTGATGCAT CAATAAATGT AACAAATCTA CAGTTGTCAG ATATTGGCAC
 351  ATATCAGTGC AAAGTGAAAA AGGCTCCTGG TGTTGGAAAT AAGAAGATTC
 401  AGCTGACAGT TCTTCTTAAG CCTTCAGGTA CAAGATGTTA TGTTGATGGA
 451  TCAGAAGAAA TTGGAAATGA CTTTAAACTA AAATGTGAAC CAAAAGAAGG
 501  TTCACTCCCA TTACTATATG AATGGCAGAA ATTGTCCAAT TCACAGAAGC
 551  TGCCCACCTT GTGGTTAGCA GAAATGACTT CACCTGTTAT ATCTGTAAAA
 601  AATGCCTCTA CTGAATACTC TGGGACATAC AGCTGTACCG TGAAAAACAG
 651  AGTGGGCTCT GATCAGTGCC TGCTTCGCCT GGATGTGGTT CCTCCTTCAA
 701  ATAGAGCTGG AACAATTGCA GGAGCTGTTA TAGGAGTTTT GCTTGCTCTA
 751  GTGCTCATTG GTCTTATTGT GTTTTGCTGT CATAAAAAGC GCAGAGAAGA
 801  AAAATACGAA AAAGAAGTGC ATCATGATAT CAGGGAAGAC GTGCCTCCTC
 851  CGAAGAGCAG AACGTCCACT GCCAGAAGCT ACCTCGGCAG CAACCACTCG
 901  TCCCTGGGAT CCATGTCTCC TTCCAACATG GAAGGCTATT CCAAGACTCA
 951  GTATAACCAG GTACCAAGCG AAGACTTTGA ACGCGCTCCT CAGAGTCCAA
1001  CTCTCCCGCT CGCTAAGGTA GCTGCCCCTA ATCTCAGCCG GATGGGAGCG
1051  GTGCCTGTGA TGATTCCAGC CCAGAGCAAG GACGGGTCCA TAGTATAA
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